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Tissue factor pathway inhibitor and the current concept of blood coagulation

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Tissue factor pathway inhibitor (TFPI) is a multivalent, Kunitz-type plasma proteinase inhibitor that regulates tissue factor-induced coagulation. TFPI directly inhibits activated factor X and, in a factor Xa-dependent manner, produces feedback inhibition of the factor VIIa/tissue factor catalytic complex. The properties of this inhibitor help explain the clinical need for 'extrinsic' and 'intrinsic' coagulation pathways and have led to a reformulation of the coagulation cascade. In the revised hypothesis, factor VIIa/tissue factor is responsible for the initiation of coagulation but, owing to TFPI-mediated feedback inhibition, amplification of the procoagulant response though the actions of factor VIII, IX and XI is required for sustained haemostasis.

Key words: Cascade, Kunitz, haemophilia, tissue factor, tissue factor pathway inhibitor, review.

Introduction

Blood coagulation is part of the haemostatic response to injury and serves to maintain the integrity of the vascular system. It involves a complex series of interactions between protease zymogens, enzymes, and cofactors that leads to the generation of thrombin and a fibrin clot. Owing to the influence of Schmidt and Morawitz, it was widely accepted in the first half of this century that the initiating event in blood coagulation was the exposure of plasma to damaged tissues. The substance in tissues responsible for this induction of coagulation was initially called tissue thromboplastin and later tissue factor (factor III). Early studies suggesting an alternative pathway of coagulation that does not require tissue factor were initially rationalized to conform to the prevalent theory of Schmidt and Morawitz. However, mounting evidence, in particular the observation that blood from haemophiliacs apparently clotted normally after the addition of tissue factor, eventually forced a re-assessment of the coagulation mechanism.

In 1964, the cascade and waterfall hypotheses proposed an intrinsic pathway of coagulation in which the exposure of the contact factors (factor XII, high molecular weight kininogen, and prekallikrein) in plasma to a surface leads to the initiation of coagulation.[1,2] Factor VIIa/tissue factor-mediated coagulation was relegated to an ancillary role since factors VIII and IX, whose deficiencies cause the severe bleeding of haemophilia, lay in the intrinsic pathway. Although the segregation of the known coagulation factors into intrinsic and extrinsic coagulation pathways and the availability of assays to test each pathway (PTT and PT, respectively) proved invaluable in the diagnosis of haemorrhagic diseases, the cascade and waterfall hypotheses failed to accurately reflect haemostasis. Individuals deficient in one of the contact factors required for the initiation of intrinsic coagulation are asymptomatic, whereas individuals deficient in factor VII bleed.

Returning to the original tenet of Schmidt and Morawitz, it is now generally accepted that tissue factor is responsible for the initiation of physiologic coagulation. Nevertheless, factor VIIa/tissue factor mediated factor X activation and thrombin generation is clearly insufficient for normal haemostasis as individuals with factor VIII, factor IX, or factor XI deficiency suffer a haemorrhagic diathesis. Presumably Biggs and colleagues were demonstrating the *in vitro* counterpart to these clinical observations when they reported many years ago that coagulation is delayed and incomplete following the addition of low concen-

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trations of tissue factor to haemophiliac plasma[2]. The properties of the rediscovered inhibitor of tissue factor induced coagulation, tissue factor pathway inhibitor (TFPI), helps to explain the requirement for factors VIII, IX, and XI in coagulation initiated by tissue factor.

Tissue factor pathway inhibitor

Although experiments performed more than 40 years ago documented the presence of an endogenous inhibitor of tissue factor-mediated coagulation, only within the past 10 years has renewed interest ultimately led to its purification and biochemical characterization.[4-10] The mature TFPI molecule is unique, with an acidic amino-terminal region followed by three tandem Kunitz-type proteinase inhibitory domains and a basic carboxy-terminal region (Figure 1).[8] The presence of multiple Kunitz-type domains in TFPI is consistent with its dual targets for inhibition, factor Xa and factor VIIa/tissue factor.

The second Kunitz domain of TFPI is responsible for factor Xa inhibition, but other parts of the molecule also appear to be involved in its interaction with factor Xa.[11] The basic carboxy-terminal region of TFPI is required for rapid and effective inhibition of factor Xa action.[12] Furthermore, proteolytic cleavage of TFPI between Kunitz domains 1 and 2 produced by neutrophil elastase dramatically reduces the ability of TFPI to inhibit factor Xa (and factor VIIa/tissue factor).[13]

TFPI's ability to inhibit factor Xa action is predominantly responsible for the prolongation of one-stage coagulation assays (including the prothrombin time, PT) when exogenous TFPI is added to plasma *in vitro*.[14] Heparin enhances TFPI's inhibition of factor Xa. The carboxy-terminal region of the TFPI molecule is required both for the potent anti-factor Xa activity of TFPI and for its heparin binding.[15] Thus, the carboxy-

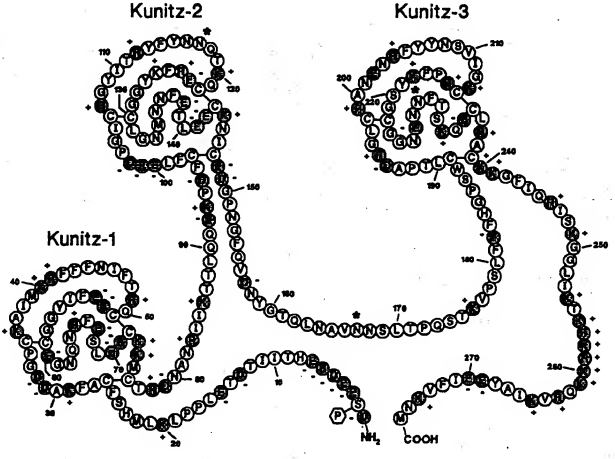

Figure 1. Structure of TFPI. The Kunitz-type domains are listed and the charges of the amino acid side chains are shown. *Potential sites for N-linked glycosylation; P, site of partial phosphorylation.

terminal truncated forms of TFPI circulating in plasma and those produced through recombinant technology possess substantially less anticoagulant activity.[16,17]

Factor Xa-dependent inhibition of factor VIIa/tissue factor by TFPI involves the formation of a quaternary complex containing factor Xa–TFPI–factor VIIa/tissue factor in which the second Kunitz domain of TFPI binds factor Xa and the first Kunitz domain binds factor VIIa (Figure 2).[9,11] This inhibitory complex could result from the initial binding of factor Xa to TFPI, with subsequent binding of the factor Xa–TFPI complex to factor VIIa–tissue factor, or alternatively, TFPI could bind to a preformed factor Xa–factor VIIa/tissue factor complex. Thus, at physiological concentrations TFPI mediates feedback inhibition of the factor VIIa/tissue factor complex, but it does not inhibit the activity of factor VIIa/tissue factor until the complex has produced some factor Xa (and factor IXa).

The requirement of factor Xa for the inhibition of factor VIIa/tissue factor by TFPI, however, is not absolute, and TFPI inhibits the activation of factor IX by factor VIIa/tissue factor in the absence of factor Xa.[18] Fifty-fold greater concentrations of TFPI, however, are needed to produce an inhibitory effect equivalent to that observed when factor Xa is present. The factor Xa-independent inhibition of factor VIIa/tissue factor by TFPI is of uncertain physiological relevance but could be important when TFPI is used as a therapeutic agent, resulting in plasma levels of TFPI many-fold greater than the levels found in normal plasma. Whereas the basic carboxy-terminus of TFPI is required for optimal factor Xa inhibition, for high-affinity heparin binding, and for a potent anticoagulant effect in one-stage coagulation assays (e.g. the PT), full-length and carboxy-terminal truncated forms of TFPI appear to produce similar factor Xa-dependent inhibition of factor VIIa/tissue factor.[12]

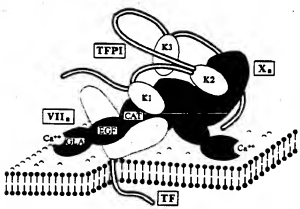

Figure 2. Factor Xa–TFPI–factor VIIa–tissue factor quaternary inhibitory complex. Factor VIIa is shown bound to tissue factor at the surface of a cell. Factor Xa is represented on the right. The Kunitz-1 domain of TFPI is shown bound at the catalytic site of factor VIIa and the Kunitz-2 domain of TFPI is shown bound at the catalytic site of factor Xa. The γ-carboxyglutamine acid (Gla), epidermal growth factor (EGF), and catalytic (CAT) domains of factor VIIa and factor Xa, as well as the Kunitz domains (K1, K2, K3) of TFPI are depicted as globular structures.

Endogenous TFPI

The plasma concentration of TFPI is low (~ 2 nM), and much of the circulating TFPI is bound to lipoproteins, including low-density lipoprotein (LDL), high-density lipoprotein (HDL), and lipoprotein (a) [Lp(a)].[19,20] Predominant forms of TFPI in plasma have molecular weights of 34 and 41 kDa, but less abundant forms of higher molecular mass are also present. This size heterogeneity of plasma TFPI reflects apparent proteolytic carboxy-terminal truncation of the molecule and its formation of mixed disulphide complexes with apolipoprotein A-II and potentially other proteins.[21] The major form of TFPI bound to LDL has a molecular weight of 34 kDa and lacks the distal portion of full-length TFPI, including at least part of the third Kunitz domain. The 41 kDa form of TFPI that circulates with HDL is apparently a similar truncated form of TFPI disulphide-linked to apolipoprotein A-II. Additional forms that have undergone less carboxy-terminal truncation and some full-length TFPI (43 kDa) also circulate in plasma. The mechanism by which carboxy-terminal truncated forms of TFPI associate with plasma lipoproteins is not yet known.

Platelets carry approximately 10% of the total TFPI in blood and release their TFPI following stimulation by thrombin and other agonists.[22] The concentration of TFPI in blood escaping from a superficial laceration (template bleeding time) increases progressively and reaches levels three-fold those of venous plasma by the time the bleeding has stopped. This additional TFPI is likely derived from platelets that aggregate at the site of the wound, although the potential contribution of other cells to the local increase in TFPI concentration cannot be excluded.

The *in vivo* infusion of heparin increases the circulating levels of TFPI in plasma two- to four-fold.[23,24] Since the *ex vivo* addition of heparin to blood or plasma does not change the TFPI concentration, the *in vivo* effect of heparin appears to be mediated by the release of TFPI from intra- or extracellular stores. The source of this additional TFPI is thought to be the endothelium, where TFPI may be bound to heparan sulphate or to other glycosaminoglycans at the endothelial surface.

TFPI is synthesized by cultured cells derived from a variety of tissues. It is not clear, however, that the pro-

duction of TFPI by these cells in tissue culture accurately reflects the sites of endogenous TFPI expression. Indeed, whereas TFPI was initially isolated from cultured HepG2 cells (human hepatoma), primary cultures of hepatocytes do not produce TFPI and a recent immunohistochemical study of human tissues detected TFPI in the endothelium of small vessels but not in hepatocytes.[25,26] Data at present suggest that the endothelium is the major *in vivo* source of TFPI and thus it is of interest that the TFPI gene contains shear-stress response elements (SSREs) which have been shown to modulate the expression of other endothelial cell products.[27]

The TFPI released by heparin *in vivo* represents the full-length molecule. This form possesses substantially greater factor Xa inhibitory activity, which is enhanced to a greater extent by heparin, than the carboxy-terminal truncated forms of TFPI that circulate in plasma. The extent to which heparin's action on the concentration and activity of TFPI contributes to the antithrombotic effect of heparin therapy remains to be defined.

Animal studies have shown that infused TFPI is predominantly taken up by the liver and kidney and recent studies suggest that the low density lipoprotein receptor-related protein/α_2-macroglobulin receptor (LRP) plays an important role in the hepatic clearance and degradation of plasma TFPI.[28,29] LRP functions as an hepatic endocytosis receptor for several other plasma proteins, including α_2-macroglobulin–protease complexes, free plasminogen activators as well as plasminogen activators complexed with their inhibitors, and β-migrating very low density lipoproteins complexed with either apolipoprotein E or lipoprotein lipase. The LRP-mediated clearance of TFPI appears to involve a two-step process in which TFPI first binds to an unrelated receptor(s), potentially glycosaminoglycans, before its transfer to LRP and subsequent internalization. This initial hepatic cell surface binding requires the carboxy-terminus of TFPI and is inhibited by clinically achievable levels of heparin.[30] Whether gp330, a cell surface receptor in kidney that is closely related to LRP, mediates the renal clearance of TFPI is under investigation.

Low levels of TFPI are occasionally seen in septicaemia and disseminated intravascular coagulation, but more often TFPI concentrations are normal.[25] The progression of disseminated intravascular coagulation in the presence of a normal plasma level of TFPI is consistent with the fact that TFPI, at physiological concentrations, inhibits factor VIIa/tissue factor effectively only after factor Xa has been generated. Thus, TFPI dampens but does not prevent the coagulation process when continuing generation of tissue factor occurs.

Animal studies have shown that the depletion of endogenous TFPI sensitizes rabbits to the disseminated intravascular coagulation induced by tissue factor or endotoxin infusion.[31,32] Conversely, the infusion of high therapeutic concentrations of TFPI, predicted to directly inhibit the factor VIIa/tissue factor complex in the absence of factor Xa, ameliorates the intravascular coagulation induced by tissue factor in rabbits and prevents mortality in a baboon model of *Escherichia coli* sepsis.[33,34]

Although TFPI deficiency might be expected to cause a prothrombotic phenotype, no individual with TFPI deficiency has yet been identified. The low plasma levels of TFPI found in abetalipoproteinaemic patients (< 25% of normal individuals) appear to simply reflect the absence of LDL, a carrier of TFPI in plasma. The total TFPI in these patients, as estimated by plasma TFPI levels following heparin infusion, is similar to that of normal individuals.[24]

The current concept of blood coagulation

The properties of TFPI suggest that it functions *in vivo* to inhibit the factor VIIa/tissue factor catalytic complex through a novel feedback mechanism and have led to the formulation of a revised hypothesis of coagulation (Figure 3).[9,10] In this scheme, coagulation is initiated when damage to blood vessels at the site of a wound exposes blood to the tissue factor produced constitutively by cells beneath the endothelium. The factor VII or VIIa present in plasma then binds to this tissue factor, and the factor VIIa/tissue factor complex activates limited quantities of factor X and factor IX. With the generation of factor Xa, the inhibitory effect of TFPI becomes manifest and prevents further production of factor Xa and factor IXa by factor VIIa/tissue factor. Further generation of factor Xa must then occur through the alternative pathway involving factor VIIIa and IXa.

The role of factor XIa in the revised hypothesis is to produce additional factor IXa to supplement that generated by factor VIIa/tissue factor, which is limited due to the presence of TFPI. Thus, in certain conditions or at certain sites where the initial quantity of factor IXa produced by factor VIIa/tissue factor is insufficient or the processes opposing coagulation (for example fibrinolysis) are particularly active, factor XIa is required for effective haemostasis. Such a scheme is consistent with the clinical picture of factor XI deficiency in which 'spontaneous' haemorrhage or haemorrhage following general surgical procedures is uncommon unless the factor XI level is severely depressed, whereas bleeding is common when trauma or surgery involves tissues such as the mouth, nose, tonsils and urinary

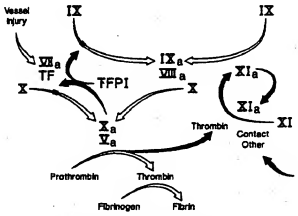

Figure 3. The revised coagulation cascade. Haemostasis is initiated when factor VII or factor VIIa in plasma gains access to tissue factor at a site of blood vessel injury. Limited quantities of factor IXa and factor Xa are generated before there is feedback inhibition of the factor VIIa/tissue factor complex mediated by TFPI in concert with factor Xa. The generation of factor Xa is then amplified through the action of factor VIIIa and factor IXa; the latter produced initially by factor VIIa/tissue factor and supplemented by factor XIa. The mechanism(s) for *in vivo* factor XI activation remains to be established.

tract with high levels of fibrinolytic activity.[35] The physiological mechanism for factor XI activation remains to be defined, but recent *in vitro* studies have shown that thrombin is capable of activating factor XI and that, in the presence of artificial polyanions (e.g. dextran sulphate or sulphatides), this process is amplified through the auto-activation of additional factor XI by factor XIa.[36–38] Nevertheless, redundant pathways for factor XI activation involving thrombin, the classical contact system (factor XII, high molecular weight kininogen, prekallikrein), or other as yet unidentified activators are clearly possible.

Present data are consistent with the notion that, in normal haemostasis, factor VIIa/tissue factor is responsible for the initial factor Xa generation which provides sufficient thrombin to induce the local aggregation of platelets and the activation of the critical cofactors V and VIII. However, the factor Xa produced by factor VIIa/tissue factor and dampened by TFPI is insufficient to sustain haemostasis and must be amplified through the actions of factors IXa, VIIIa, and in certain circumstances, XIa for ultimate and persistent local haemostasis. This requirement presumably reflects the removal of activated coagulation factors by the flowing blood and their inactivation by protease inhibitors, the effect of endogenous anticoagulant mechanisms (e.g. activated protein C), and the competing process of fibrinolysis.

Based on the properties of TFPI and the revised coagulation cascade, it was suggested several years ago that the inhibition of TFPI action might provide a novel means for the amelioration of bleeding in haemophiliacs, particularly those with inhibitors.[5] For such a strategy to be successful, TFPI inhibition would need to be rapid and potent as both the TFPI circulating in plasma and that locally released by activated platelets would require inactivation. Furthermore, the rate/extent of factor Xa and subsequent thrombin generation produced by factor VIIa/tissue factor at the site of a wound would need to be sufficient to overcome the effects of their endogenous inhibitors. Although a potent activator of factor X, the factor VIIa/tissue factor complex is kinetically much less efficient than factor IXa/factor VIIIa.[39] Moreover, the presumed supplemental factor IXa produced by factor XIa in normal individuals at sites with low levels of tissue factor exposure would be of no consequence in haemophiliacs lacking factor VIII or factor IX.

Despite these caveats, Erhardtsen and colleagues have recently shown that TFPI inhibition does indeed reduce bleeding in an animal model of haemophilia A.[40] In their experiments, rabbits were made severely deficient in factor VIII by the infusion of specific antibodies and were then treated or not treated with the infusion of anti-TFPI antibodies prior to undergoing the haemostatic stress of a cuticle laceration. Treatment with anti-TFPI antibodies significantly shortened the bleeding time in these factor VIII deficient rabbits, although not to the same level as the bleeding times found in normal rabbits. Unfortunately the effectiveness of TFPI inhibition produced *in vivo* was not defined, thus it is not clear if the residual prolongation of bleeding times in treated animals compared with normal controls was related to residual TFPI activity at the wound site or properties inherent to the factor VIIa/tissue factor complex, for example its concentration at the site of injury and its limited rate of factor X activation. Certainly before TFPI inhibition can be considered as a potential therapeutic modality for haemophilia similar experiments must be performed in haemophiliac animals with congenital deficiencies. As factor IX and factor X are competing substrates for factor VIIa/tissue factor catalytic activity, it is conceivable that such therapy would be more efficacious in factor IX, rather than factor VIII, deficiency. Nevertheless, these experiments serve to underscore the *in vivo* importance of TFPI as an endogenous regulator of tissue factor induced coagulation.

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